Original Research Article

Phenotyping and Whole Cell Protein Profiling of *Edwardsiella tarda* strains isolated from infected Freshwater Fishes

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A B S T R A C T

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A total of thirtyfive isolates of *Edwardsiella tarda*, isolated from infected rohu (*Labeo rohita*) and catla (*Catla catla*) were successfully differentiated into three distinct strains based on their biochemical properties. The percentage similarity of phenotypic characteristic of the present strains including reference strains (ATCC 15947) ranged from 73.3% to 100%. The whole cell protein (WCP) profiling of these strains by using SDS PAGE yielded 12–16 polypeptide bands. However, common bands of 20.3, 42.7, 53.4 and 93.5 kDa was found in all the three strains as well as in reference strain. Rabbit polyclonal antibodies (RPAbs) raised against WCP of ATCC 15947 strain of *E. tarda* detected common protein bands of 30.5 kDa and 42.7 kDa in all strains and two more band of 53.4 and 66.4 kDa in ATCC 15947 strain by western blotting. These polyclonal antibodies strongly reacted with WCP of all other strains of *E. tarda* by dot-ELISA test but they showed no cross reactivity with WCP of other bacteria i.e *Aeromonas hydrophila, Vibrio alginolyticus*. This study indicated that RPAbs against WCP is useful for rapid and confirmatory detection of *E. tarda* by Dot ELISA.

Introduction

The outbreak of infectious diseases acts as significant setback for successful aquaculture production and trade in India and elsewhere (Yunxia et al., 2001). Bacteria are among the highly encountered causes of diseases in cultured warm water fish. Out of the most annihilating bacteria *Edwardsiella tarda*, act as a predominantly enteric pathogen of both fresh water and brackish water fishes (Plumb, 1994). It is mainly responsible for causing a serious systemic bacterial disease called as edwardsiellosis, having a worldwide distribution and affecting a variety of fish taxa (Austin and Austin, 1999; Maiti et al., 2009). The bacterium act as a pathogen with wide range of hosts other than fish such as reptiles, birds, mammals including humans (Plumb 1993; Park and Oh, 2008). It is the aetiological

agent of several pathologic symptoms of fresh water and marine fish, particularly causing entero hemorrhagic septicemia (Benli and Yildiz, 2004). This disease had a disastrous effect on fish culture, occurred in northern and southern Europe in turbot in recent years (Castro et al., 2006). The losses attributed to *E. tarda* infection in cultured eel in Japan for the year 1984 was about 815 Yen (Meyer and Bullock, 1973). Due to this edwardsiellosis has been known as a disease of primary importance and required a great attention for successful aquafarming.

At present, the information on phenotypic, genotypic and proteotypic characteristic of *E. tarda* from both environmental and clinical, are lacking. Thus, the present study was conducted to reveal phenotypic and proteotypic characteristic of *E. tarda* isolated from freshwater fishes. The phenotypic study of *E. tarda* was carried out through biochemical studies which help in the identification of bacteria. The proteotypic study on WCP was developed to standardize protein based markers for easy and quick diagnosis of disease caused by these bacteria.

The WCP were further used as antigen to raise polyclonal antibodies in rabbit and the immunogenic protein component were detected through western blotting assay. The specificity and sensitivity of polyclonal antibodies were also examined through dot-ELISA to develop another specific diagnostic assay for detection of *E. tarda.* These informations are not only useful for bacterial disease diagnosis purpose but also to enable the development and formulation of appropriate and effective subunit vaccines against this pathogen.

Materials and Methods

Isolation and identification of *Edwardsiella tarda*

A total of thirty five isolates representing three different strains of *E. tarda* and the reference strains (*E. tarda* ATCC 15947) were used in the present study. These isolates were collected during the month of June-September 2010 from edwardsiellosis infected 18 numbers of rohu (*Labeo rohita*) & 9 numbers of catla (*Catla catla*) weighing around 500 g, obtained from ponds of Central Institute of Freshwater Aquaculture, Bhubaneswar. All the fishes collected were having symptoms like small hemorrhages and necrosis lesions externally. Internally abundant ascitic fluids were found in their abdominal cavity. For bacteriological analysis swabs were taken from the infected parts of the fishes. After pre-enrichment in tryptone soya broth (TSB; HiMedia, India), the bacteria samples were streaked on Rimler Shotts Agar (RS Medium; HiMedia, India) for their presumptive identification and incubated at 37°C for 24 h. Well differentiated single bacterial colony was further streaked onto tryptone soya agar (TSA; HiMedia, India) for obtaining pure culture.

Biochemical analysis

For morphological study the pure cultures of the isolates were subjected for Gram staining and viewed microscopically. Further conventional biochemical tests like oxidase, catalase, methyl red, Voges-Proskauer, indole, citrate utilization test, gelatinase, amylase, lecithinase, caseinase, haemolysin production, various sugar utilization test, triple sugar iron agar test, were performed for the identification as well as strain differentiation of bacteria

and results were compared with the reference strain of *E. tarda* (ATCC 15947; Fang et al., 2006).

Numerical taxonomy of the present strains

A data matrix was prepared basing on the biochemical tests of the present strains and reference strain. The positive and negative results of biochemical tests of all the strains were scored as 1 and 0, respectively. The binary matrix data were then analyzed with the unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal, 1973). Using DendroUPGMA program, Dice coefficient (S) similarity matrixes were then calculated. The distance (d) between two strains is calculated with the formula d = 1 − S. A 'd' value of 0 indicates that the two strains have identical properties. This method was mostly used for the detection of interrelationships between different strains of same bacteria. Further the similarity of the phenotypic characters among the different strains was also calculated by the formula given by Dice (1945) which is as follow:

Percentage of similarity, F= 2Nab X 100% / Na + Nb
Where,
Nab=Number of shared similar phenotypes
Na= Total number of positive reactions in lane A
Nb= Total number of positive reactions in lane B

Extraction of WCP

WCP of the present strains were extracted by the method given by Simpson (2003) with slight modification. Overnight broth culture of bacterial strains were prepared using TSB and incubated at 37°C for 24 h. The cultured were then formalin inactivated at concentration of 0.2% and kept for 24 h at 4°C. The WCP was obtained by centrifugation at 2800 rpm for 45 min at 4°C. The supernatant was discarded and the pellet was suspended in the phosphate buffer saline (PBS, pH 7.2). It was further subjected for centrifugation at 5000 rpm for about 15 min. Finally the pellet obtained was sonicated at 45 Hz for 10 min. The sonicated product was centrifuged at 10,000 rpm for 15 min and the supernatant product obtained was the WCP antigen. It was stored at -20°C for future analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) analysis

The WCP profiles of three strains of *E. tarda* and reference strain ATCC 15947 were analyzed by SDS PAGE as described by Laemmli (1970). Samples were mixed with 2X SDS gel loading buffer in 1:2 ratio and were then heated at 100°C for 5 min. Electrophoresis of protein sample was carried out using 12% separating gel and 4% stacking gel in the Mini protein tetra electrophoresis cell (Bio-Rad, USA). Electrophoresis was carried out at 120 V until the bromophenol blue dye font reaches the bottom of the gel. The gel was then stained with Coomassie Brilliant Blue R-250 followed by destaining. The molecular weights of the proteins were determined with standard molecular weight marker (Banglore Genei, India) using Alpha Inotec Software.

Raising of polyclonal antibodies in rabbit (RPAbs)

Hemolysin positive *E. tarda* ATCC 15947 strain was selected to raise polyclonal

antibodies in a New Zealand white rabbit weighing around 1.5 kg. Rabbit was injected intramuscularly (on the hind leg) with emulsion of Freund's complete adjuvant (FCA) and 150 µg of WCP antigen of *E. tarda* ATCC 15947. The animal was given with a booster dose on 14th and 28th days of immunization with same dose of emulsion containing WCP antigen and Freund's incomplete adjuvant (FIA). Blood from rabbit was collected by ear vein puncture on 42nd days of post immunization. It was allowed to clot at room temperature for 2-3 h. Serum was then collected by centrifugation of the clotted blood at 5000 rpm for 10 min and was stored at -20°C for further use.

Western blotting of WCP

Western blot immunoassay was performed to determine the reactivity of the rabbit antiserum to WCP antigen. The WCP obtained from three strains as well as reference strains of E. tarda were first electrophoresed by SDS-PAGE using the standard protocol mentioned above. After electrophoresis the gel was subjected for electrotranfer onto a nitrocellulose membrane (pore size 0.45 µm; Millipore, USA) in transfer buffer, as described by Towbin et al. (1979). After the transfer, the membrane was washed in deionized water and blocked with 5% skimmed milk powder solution in phosphate buffer saline containing 0.05% Tween-20 (PBS-T) at 37°C for 1 h. Membrane was then incubated with the primary antibody at 1:20,000 dilution at 37°C for 1 h, washed three times in PBS-T and again incubated with secondary antibody i.e. HRP conjugated goat anti-rabbit IgG (Jackson, USA) for 1 h at 37°C. Following further washes in deionized water, bound antibodies were detected by addition of 3,3',5,5'-tetramethylbenzidine membrane peroxidase substrate (KPL, USA) and the color reaction was stopped by rinsing to the membrane extensively with deionized water.

Dot-ELISA for determination of specificity of RPAbs

Dot-ELISA test was performed as per the method given by Swain et al. (2001) with slight modifications. Nitrocellulose paper (NCP) strip was coated separately with 3 µl of WCP antigen of all three strains and reference strain of *E. tarda*. The NCP was also coated with WCP of *A. hydrophila* ATCC 14940 and *V. alginolyticus* ATCC 17749, taken as negative controls. The coated NCP strip was dried at room temperature for 2 h. Then, the strips were blocked in PBS-T containing 5% skimmed milk powder at 37°C for 30 min followed by washing thrice with PBS-T and incubated with primary antibody (1:400 dilutions) for 1 h at 37°C. Then it was washed several times in PBS-T and incubated further with secondary antibody i.e. anti rabbit horse radish peroxidase conjugate (Vector Lab, USA) for 30 min. The NCP was again incubated with Anti-Bovine-Complex (ABC) reagent for 30 min. Lastly, NCP was transferred into a new plate and then incubated with 3-amino-9 ethyl carbazole (AEC) reagent, for 10 min in dark. The NCP strip was washed properly with distilled water, air dried at room temperature and blots were detected.

Results and Discussion

Presumptive identification of bacteria was carried out from their colony morphology over the RS plate. They form typical greenish colonies with black center over the RS plate. Under microscope Gram negative small rods were observed. In the

present study, based on biochemical results a total of three different strains of *E. tarda* were obtained out of thirtyfive isolates (Table 1). All the strains were positive for motility, catalase, methyl red, indole test, production of acid and gas from glucose, maltose and fructose, hydrogen sulphide production on TSI agar slant, decarboxylation of lysine and ornithine but exhibit negative reactions for oxidase, Voges-Proskauer test, decarboxylation of arginine, production of acid from lactose, rhamnose, sucrose, mannitol and adonitol. However variable results were obtained in other biochemical test within the strains of *E. tarda*. All the biochemical results were compared with the reference strain ATCC 15947 (Table 1).

Numerical analyses of 30 different biochemical traits of all four strains of *E. tarda* were carried out to establish the percentage similarity between the strains. The percentage similarity of the biochemical characteristic of the present strains and reference strain ranged from 73.3% to 100% (Table 2). Strain ET10 and ET12 shared 73.3% similarity of biochemical characteristic. Strain ET 10 and ET 14 exhibit 85.7 to 100% similarity. Comparison percentage of similarity from reference strain to ET 10, ET-12, and ET 14 ranged from 77.4% to 90.9% and the relation between all three strains with ATCC 15947 was represented in Fig. 1.

One-dimensional SDS-PAGE of WCP of three strains including reference strain revealed protein profiles containing discrete bands with molecular weights ranging within 10.5-162.7 kD. The number of protein bands and molecular mass of corresponding bands varied in different strains. However, protein bands with molecular weight of 20.3, 42.7, 53.4,

93.5 kD were found common in all the strains. ATCC 15947 showed 16 discrete protein bands of molecular weights ranging from 10.5-151.9 kD (Fig. 2). ET 10 showed 13 distinct bands with molecular weight of 14.1-101.3 kD. ET 12 and ET 14 showed 12 distinct protein band of molecular weight 14.1-107 kD and 11.5-162.7 kD respectively.

In western blotting, the RPAbs detected 4 protein bands of WCP of *E. tarda* ATCC 15947 similar to SDS-PAGE at 30.5, 42.7, 53.4 and 66.4 kD (Fig. 3). This indicated that four out of sixteen protein bands are antigenic. A most interesting feature seen in western blotting was that the 42.7 and 30.5 kD protein bands were main antigenic proteins present in WCP of all our *E. tarda* strains (Fig. 3). Protein band of molecular weight 53.4 kD was also found in ET 14 strain.

In Dot-ELISA test, brown coloration was developed on the NCP strip coated with ATCC 15947 and other strains of *E. tarda* due to strong reaction of antigen with the homologous antiserum but no color development was seen with the WCP of *A. hydrophila* ATCC14940 and *V. alginolyticus* ATCC17749; suggesting that the test is very specific for identification of bacteria (Fig. 4).

E. tarda has been considered as a re-emerging pathogen in both marine and freshwater aquaculture. It is a common pathogen, which has been isolated from farmed fish such as eel, tilapia, flounder, sea bream, stripped bass, catfish and carps (Meyer and Bullock, 1973; Yasunaga et al., 1982; Sae-qui et al., 1984; Herman and Bullock, 1986; Han et al., 2006; Yu et al., 2009; El-Yazeed and Ibrahem, 2009). It is mainly responsible for causing a significant disease edwardsiellosis or

Table.1 Comparison of phenotypic characteristics between *E. tarda* strains isolated and reference strain (ATCC 15947).

Characteristic	ET10 (12isolates)	ET12 (9 isolates)	ET14 (14isolates)	ATCC 15947	Characteristic	ET10 (12isolates)	ET12 (9 isolates)	ET14 (14isolates)	ATCC 15947
Gram Stain	-	-	-	-	Caseinase	-	+	-	+
Oxidase	-	-	-	-	Haemolysin production	-	+	-	+
Catalase	+	+	+	+	Lactose, acid	-	-	-	-
Motility	+	+	+	+	Galactose, acid	-	+	+	+
Methyl red	+	+	+	+	Maltose, acid	+	+	+	+
Citrate	+	-	-	+	Fructose,acid	+	+	+	+
Arginine dihydrolase	-	-	-	-	Arabinose, acid	-	+	-	+
Ornithine decarboxylase	+	+	+	+	Mannose, acid	-	+	+	-
Lysine decarboxylase	+	+	+	+	Rhamnose, acid	-	-	-	-
Voges-Proskauer	-	-	-	-	Sucrose, acid	-	-	-	-
H$_2$S Production	+	+	+	+	Mannitol, acid	-	-	-	-
Indole	+	+	+	+	Adonitol, acid	-	-	-	-
Gelatinase	-	-	-	-	Raffinose, acid	+	-	+	-
Amylase	-	-	-	+	Sorbitol,acid	-	-	-	-
Lecithinase	+	-	-	-	Triple sugar iron	K/AG	K/AG	K/AG	K/AG

*ET = *Edwardsiella tarda,* + = positive reaction, - = negative reaction, K = alkaline, A = acid, G = gas.

Table.2 Percentage similarity of phenotypic characteristics of present strains

	ET10	ET12	ET14	ATCC15947
ET10	100			
ET12	73.3	100		
ET14	85.7	86.7	100	
ATCC15947	77.4	90.9	77.4	100

Fig.1 Dendrogram based on UPGMA showing showing maximum similarity between ET10 and ET14 as well as ET12 and ATCC15947 derived from their phenotypic characterization
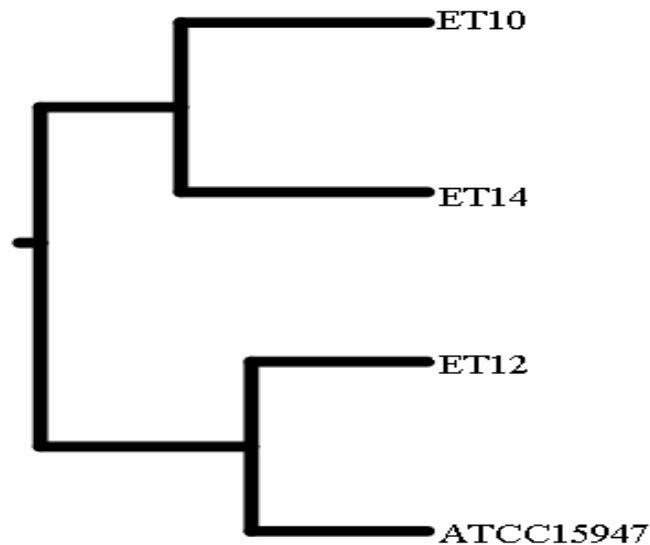

Fig.2 SDS-PAGE analysis of Whole Cell Protein of *E. tarda* strains. From left to right lane 1: Molecular weight protein marker (97.4- 14.4 kDa); lane 2: WCP of ATCC 15947; lane3: WCP of ET14; lane 4: WCP of ET12; lane 5: WCP of ET10
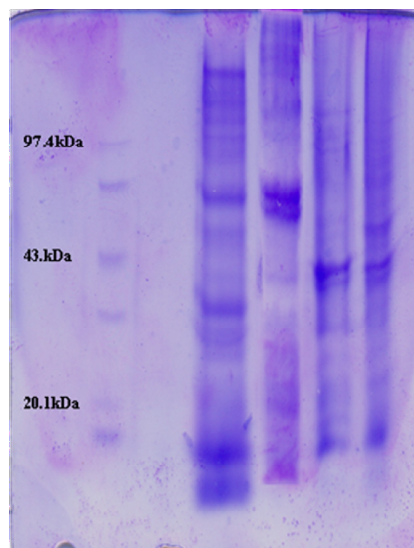
Fig.3 Western blotting profile of WCP of *E. tarda* . From left to right WCP of ATCC 15947 (3), cross reacted WCP of ET12 (1), ET14 (2) and ET10 (4) with rabbit serum
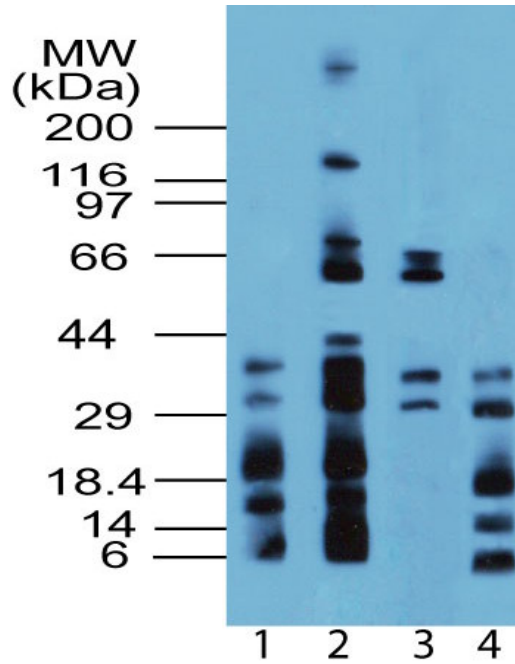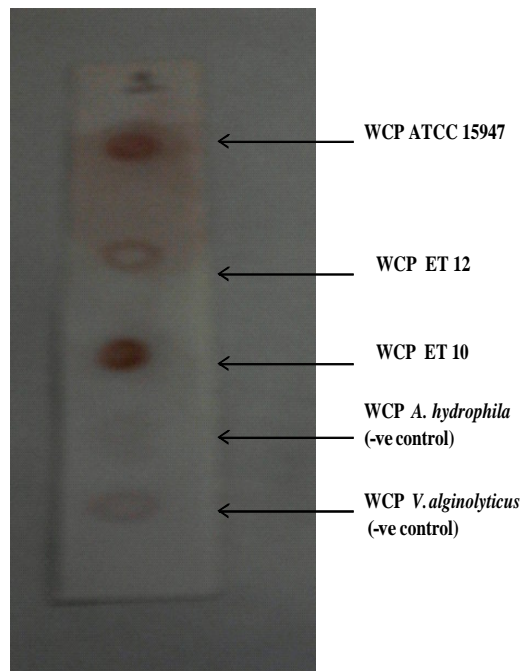

Fig.4 Detection of *E. tarda* specific antigen through dot ELISA analysis, showing positive reaction (with deep brown colour) for WCP of ATCC 15947, ET12 and ET10.

emphysematous putrefactive disease (EPD) that causes mass mortality in freshwater fishes (Keskin et al., 2004; Galal et al., 2005; Esteve et al., 2006). This disease is considered of highly economic importance as it lower the fish marketability due to bad appearance and unacceptable putrid odour produced by the bacteria (Noga 2000). However a few comparative characterization studies among the isolates of *E. tarda* with diverse host and geographical origin have been carried out. So, the present study was carried out to reveal phenotypic and proteotypic characteristic of *E. tarda* isolated from disease infected freshwater fishes i.e. from rohu and catla. This study was important not only from epidemiological point of view but also for implementing effective preventive measures against this pathogen.

The gross sign displayed by the fish in the present study included abdominal distension (ascitic fluid), local hemorrhagic lesions on the skin, which are similar to those reported by several authors (Han et al., 2006; Podros et al., 2006).

The presumptive identification of bacteria has been carried out from their colony morphology over RS plate, which acts as a selective medium for *E. tarda* (Buller, 2004; Achrya et al., 2007). Biochemical characterizations have proved to be a valuable method for typing and differentiation of bacterial fish pathogen (Tison et al., 1982; Austin et al., 1997). In the present study, 30 different conventional biochemical tests have been carried out for proper identification and strain differentiation of *E. tarda* isolates. Most of the phenotypic characteristic of the isolates were similar as claimed in Bergey's manual of determinative

bacteriology (Holt et al., 1994). Based on the biochemical test results three different strain of *E. tarda* were obtained and were compared with the *E. tarda* reference strain ATCC 15947. However, the result obtained in biochemical profile study presented a low degree of discrimination among the strains because all the strains exhibit similar results in most of the biochemical test. Heterogeneity among them were found in few biochemical tests i.e. citrate utilization test, fermentation of various sugars like galactose, arabinose, raffinose, mannose, production of hemolysin, amylase,lecithinase and caseinase. The above finding exhibit similarity with the findings of Amandi et al. (1982), Wei and Musa (2008) and Kumari (2011). According to Baya et al. (1997) and Stock and Widemann (2001) all the isolates failed to utilize mannose and rhamnose but two of the present strain ET-12 and ET-14 were able to ferment mannose and all the strains exhibit negative reaction towards rhamnose utilization. Variations were found in terms of citrate utilization test in our study. On contrast all the isolates in the studies of Coles et al. (1978) and Baya et al. (1997) failed in citrate utilization test. According to Williams and Lawrence (2005) the haemolysin is an important virulence factor of many species of bacteria including *E. tarda*. Among all the strains ATCC 15947 and ET12 exhibit beta hemolysis activity over the blood agar medium. So, they seem to be more virulent as compared to other strains. Furthermore, Hirono et al. (1997) describe the ability of *E. tarda* to perform hemolysis activity against red blood cells as it posses two types of genes Eth A and Eth B. These variations in phenotypic characteristic among *E. tarda* may be due to presence and absence of plasmid that control the metabolic of the phenotypic characteristics

of the *E. tarda* (Achrya et al. 2007; Kumari 2011).

The numerical taxonomy study of biochemical test showed the highest ranging percentage similarity among the different strains of *E. tarda* (Wei and Musa, 2008). WCP profiling of bacteria analysis through SDS PAGE provide a rapid, simple and powerful tool for discriminating and differentiating various strains of bacteria. Ricciardi et al. (2005) characterized SDS PAGE profiles of WCP of sixty eight strains and successfully discriminated them. Further Panagala et al. (2006) showed that Whole cell protein analysis through SDS PAGE was able to differentiated nine and eighteen isolates of *E. tarda* and *E. ictaluri* respectively. In our present study the banding profiles of the Whole cell protein was able to differentiate four strains of *E. tarda* including reference strain. However, a few common bands of 20.3, 42.7, 53.4, 93.2 kDa were found in all the strains. This study collaborated with the finding of Maiti et al. (2009) who reported eight bands having molecular weight range of 38-83 kDa were present in all twenty seven isolates of *E. tarda*.

Through Western blotting four immunogenic bands with molecular weight of 30.5, 42.7, 53.4 and 66.4 kD were obtained by using polyclonal rabbit antiserum against the WCP of hemolysin positive *E. tarda* strain ATCC 15947. Out of which two most prominent antigenic bands of molecular weight 30.5 and 42.7 kD were found common in all the strains of *E. tarda.* These bands seem to be highly immunogenic and hence can be used for the production of subunit vaccine against this pathogen. This is in contrast to Japanese strains of *E. tarda* where 37 kD protein was the most prominent antigen reacting to polyclonal antibody against the formalin killed cells of *E. tarda* EF-1 (Tu and Kawai, 1998).

The diagnosis of edwardsiellosis based on clinical signs, histopathology followed by elaborate laboratory procedures and characterization of bacterial isolates are not only time consuming but also expensive (Sahoo et al., 2000). At present the ELISA technique is widely used for fish disease diagnosis (Rodak et al., 1993; Swain and Nayak, 2003). Swain et al. (2001) have used dot ELISA for rapid and confirmatory identification of *E. tarda* infected and dead fish by using the hyper immune rabbit sera against the whole cells of *E. tarda.* In our study the antibody raised against WCP of ATCC 15947 strain showed positive response to all the strains tested. But it didn't exhibit any cross reactivity with other group of bacteria like *A. hydrophila* and *V. alginolyticus.*

Since the diagnosis of a particular infection depends on the detection of its causative agents so the present study is helpful for diagnosis of significant fish pathogen *E. tarda* and formulation of effective strategies for immunization to prevent the spreading of disease in both cultured and wild freshwater fishes.

Acknowledgement

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